BINOD BIHARI MAHTO KOYALANCHAL UNIVERSITY

SYNOPSIS ON

TOXIC EFFECTS OF HYDROGEN PEROXIDE (H_2O_2) AND AMELIORATION OF VITAMIN C ON FRESH WATER FISH *Catla catla* (Ham.).

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Toxic effects of Hydrogen Peroxide (H_2O_2) and Amelioration of Vitamin C on fresh water fish *Catla catla* (Ham.).

1. INTRODUCTION:

HYDROGEN PEROXIDE

Hydrogen peroxide is the chemical compound with formula H_2O_2. It is pale blue [1] liquid, slightly more viscous than water. Hydrogen peroxide is highly reactive, strong oxidizing and bleaching (whitening) agent. It has numerous non-medical and medical uses.

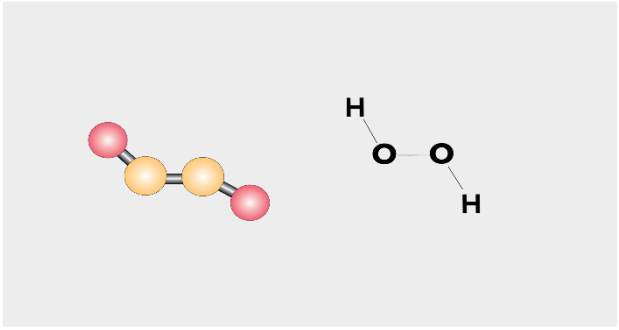

Figure 1- Structural diagram of Hydrogen Peroxide

When it is added to water, hydrogen peroxide breaks down into oxygen and water, and the formation of these by-products is one reason that hydrogen peroxide is considered as safe for the environment.

The most known use of hydrogen peroxide is as disinfectant (solution 10-30% w/w) against bacteria and viruses in homes and hospitals. Other popular use is to bleach the hair. Hydrogen peroxide is used in different industrial process, especially as blenching and disinfecting agent. In the production of paper is used to blench the wood pulp, similar usage has on the textile production. It is added to detergent due its blenching potential. Hydrogen peroxide is using in the wastewater treatment to oxidize phenols and other chemical compounds.

Hydrogen peroxide is simplest peroxide (compound with single oxygen–oxygen bond).

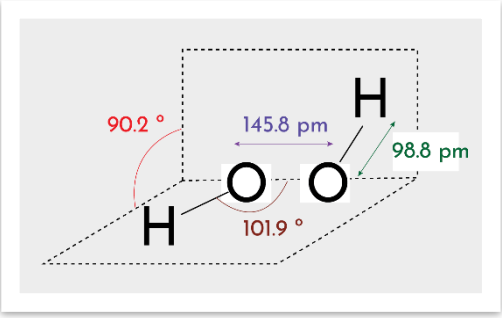

Figure 2-Hydrogen Peroxide structure

Hydrogen peroxide is a light blue to colorless and odorless liquid. Its density is 1.450 g mL^{-1}. Its boiling (decomposition) point is 150.2 ºC. Hydrogen peroxide is miscible with water. Generally, it is commercialized as 30% (w/w) solution. The density of this solution is 1.11 g mL^{-1}.

Hydrogen peroxide diluted is safe and approved by the Food and Drug Administration as antimicrobial agent. However, when concentrate; hydrogen peroxide can irritate the skin, eyes and other mucous membranes. It explodes violently when heated and the contact with combustible materials or metals such as copper, iron, nickel or zinc, may result in ignition.

It is disinfectant for cleaning wounds, and is used in aquaculture against many different disease-causing organisms, including external parasites, bacteria, fungi and on different species of fishes [2].

Hydrogen peroxide is commonly used around the world in a variety of commercial, industrial, medical, environmental, and personal hygiene applications. It is widely used in contemporary industry as a chemical intermediate in manufacturing processes, but the greatest volume of use is as a bleaching agent in the textile, pulp, and paper industries [3].

Hydrogen peroxide (H_2O_2) is a weak acid, colorless liquid, miscible with water in all proportions. It is the simplest peroxide (molecules containing two oxygen atoms covalently bonded to one another) and is commercially available in aqueous solution over wide concentration range.

In nature, hydrogen peroxide is found as a defense agent produced by some species of beetles. Moreover, hydrogen peroxide has been found as a chemical compound of the immune system of zebrafish, where the quantity of peroxide is increased after some damage. Additionally, hydrogen peroxide has showed activity as a signaling molecule in some biological processes.

VITAMIN C

Vitamin C is a water soluble vitamin needed for growth and repair of tissues in all parts of the body. It is necessary to form collagen, an important protein used to make skin, scar tissue, tendons, ligaments, and blood vessels. Vitamin C is essential for the healing of wounds, and for the repair and maintenance of cartilage, bones and teeth. Vitamin C is very important essential nutrient that we must obtain from our diet.

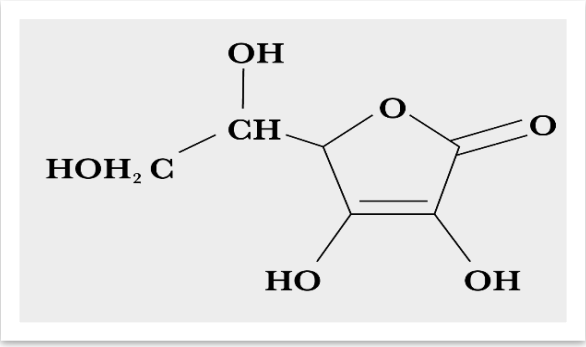

Figure 3-Structure of Vitamin C

Vitamin C or ascorbic acid (AsA) is a naturally occurring organic compound with antioxidant properties, found in both animals and plants. It functions as a redox buffer which can reduce, and thereby neutralize, reactive oxygen species. It is a cofactor for enzymes involved in regulating photosynthesis, hormone biosynthesis, and regenerating other antioxidants; which also regulates cell division and growth, is involved in signal transduction ,and has roles in several physiological processes, such as immune stimulation ,synthesis of collagen, hormones, neurotransmitters, and iron absorption, has also roles in detoxifying the body of heavy metals [4].

Vitamin-C (ascorbic acid) is an antioxidant, a free-radical scavenger, capable of decreasing or preventing the oxidation of the molecules. During oxidation reactions, transfer of electrons from substances to an oxidizing agent, some free radicals are produced, which starts chain reactions that damage animal cells. Antioxidants slow down these chain reactions by removing free-radical intermediates and eventually inhibit other oxidation reactions by being oxidized themselves.

Fish play an important role in nutrient cycles because they store a large proportion of ecosystem nutrients in their tissues, transport nutrients farther than other aquatic animals and excrete nutrients in dissolved forms that are readily available to primary producers. About 94% of all freshwater fisheries occur in developing countries [5]. They provide food and a livelihood for

millions of the world's poorest people, and also contribute to the overall economic wellbeing by means of export commodity trade, tourism and recreation [6]. It is estimated that freshwater fishes make up more than 6% of the world's annual animal protein supplies for humans.

2. REVIEW OF LITERATURE:

- Rach, J.J. [7] and coworkers reported that when fishes were exposed to hydrogen peroxide concentrations ranging between 100 to 5,000 µL/L (ppm) for 15-min and 45-min treatments every alternate day to determine the sensitivity of various species and life stages of fishes. Except for walleye (*Yellow pickerel)*, most species of the fish tested (less than or equal to 2g) tolerated hydrogen peroxide of 1,000 µL/L or greater. Walleyes (*Yellow pickerel)* were sensitive to hydrogen peroxide concentrations as low as 100 µL/L. Correlations were found between the toxicity of hydrogen peroxide and life stages of rainbow trout (*Oncorhynchus mykiss*), large fishes were more sensitive. In general, the toxic level of hydrogen peroxide increases for all species of fishes as water temperature increases.

- Vera and Migand [8] have reported that H_2O_2 caused significant differences in the 2induction of both physiological and oxidative stress responses in *Salmo salar*. Glucose and lactate were higher in the treated fish during daytime whereas cortisol levels appeared to be systematically increased (>1000 ng/mL, ng/ml=nanogram per millilitre) after H_2O_2 treatment irrespective of exposure time, although differences with control levels were higher during the day. In liver, gene expression of antioxidant enzymes displayed daily rhythmicity in both treated and control groups and showed higher mRNA expression levels in salmon treated with H_2O_2 at ZT6 (6 h after lights onset).

- Kuznetsov [9] reported Hydrogen peroxide (H_2O_2) was administered to experimental rats at the level of LD100. The indices of external breathing, blood pressure, ECG, heart thrust were determined. It was found that the increasing tonus of the vagus nucleus was

an essential feature of the pathogenesis of HP poisoning, inducing a series of pathological changes in animals.

- Multiple papers have described high (usually ≥50 μM) levels of H_2O_2 as being cytotoxic to a wide range of animal, plant and bacterial cells in culture, although LD_{50} values and the mode of cell death induced (apoptosis or necrosis) depend on the cell type used, its physiological state, length of exposure to H_2O_2, the H_2O_2 concentration used, and the cell culture media employed [10] [11] [12]. It is therefore widely thought that H_2O_2 is very toxic *in vivo* and must be rapidly eliminated, employing enzymes such as catalases, peroxidases (especially glutathione peroxidases) and thioredoxin-linked systems [13] [14] [15] [16] in Human.

- Mirvaghefi and coworker [17] showed that SOD (Superoxide dismutase) and CAT (Catalase) measurements of these and two enzymes on vitamin C could decrease and moderate the activity level. In fact, the moderation of enzymes activity level were mentioned due to the role of this vitamin is neutralizing superoxide anion free radical O2-, which resulted as decrease of activities of these enzymes. Vitamin C increases the total antioxidant capacity, and decreases its cellular damages caused by destruction of lipid peroxidation process in the presence of diazinon (Organ phosphorus insecticides) on *rainbow trout*. In general, ascorbic acid has biologically acted as decreasing and balancing biochemical reactions during oxidative stresses. Vitamin C decreases the rate of reactive oxygen species (ROS) produced in various physiological functions [18]. This vitamin has electro- negativity acted as reviver, inhibitor and scavenger of reactive oxygen species. The ascorbatic form of vitamin C, can neutralize and revive oxiradicals through electro-negativity up to two levels. In this way, compound has moderating effect on enzymatic antioxidant defense activities [19].

- Dietary antioxidants can decrease atherosclerosis in LDL (low density lipoprotein)-receptor deficient mice, cholesterol fed rabbits and cholesterol fed primates, [20][21][22]. For example, animals can be fed high cholesterol diets with induction of atherosclerosis, which was decreased by antioxidants including vitamins C and E and probucol [23] [24]

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- Mohamed *et. al.*,[25] have reported that supplementation of vitamin C in the diet of *Oreochrmis niloticus* tended to stimulate antioxidant status and immunity , improves kidney function and ameliorates the histopathological alterations induced by the exposure of renal and muscle tissue of aquatic animals than the usage of engine oil ; By performing molecular, biochemical and histopathological studies on the ameliorative effect of vitamin C on the renal and muscle tissues of Nile tilapia fish (*Oreochromis niloticus*) affected by the usage of engine oil.

- Ugur Yavuz and Yusuf Bozkurt, [26] showed that addition of ascorbic acid to the extenders (E) composing such as (E-1) 300 mM glucose, 10% egg yolk and 10% DMSO and (E-2) 0.6 mM sucrose and 10% DMSO improved rainbow trout (*Oncorhynchus mykiss*) sperm motility resulting higher fertilization of the egg.

- Wang and coworker [27] have reported that *Anoectochilus roxburghii* (a popular folk medicine in many Asian countries used in treatment of diseases such as diabetes, tumors, hyperlipemia and hepatitis) flavonoids (ARF) protects LO_2 (Liquid oxygen) cells against H_2O_2-induced oxidative stress and exerts the potent anti-aging effects in D-gal aging mice model, which may be related to the inhibition of oxidative stress. Flavonoid compounds may contribute to the anti-oxidative capability and modulating aging.

3. PROBLEMS TO BE INVESTIGATED:

In the proposed thesis we aim to investigate the following in fresh water fish *Catla catla*:

1) To study the effects of H_2O_2 in Brain, Liver, Kidney, Testis and Ovary.
2) To study the protecting role of vitamin C in Brain, Liver, Kidney, Testis and Ovary.
3) To study Histopathological, Enzymological and Biochemical test i.e., Total Protein, Cholesterol and Creatinine of fresh water fish *Catla catla*.

4. OBJECTIVES AND HYPOTHESES:

This fish is selected for study because this is one of the important surface feeder and easily available for laboratory study. This fish lives in most moderate environment if studied ecologically and is very important dietary fish for the sources of proteins.

As for to our knowledge less works or no works available on such type of topic, that there has been regarding the hazardous effects of H_2O_2 on aquatic organisms and neutralization of these effects, so the present work was to find out the possible toxic effects of H_2O_2 on freshwater fish *Catla catla* in relation to their body weight, gonadosomatic indices, enzymological studies in liver and kidney, biochemical in brain, liver, kidney, testis, ovary and histopathological changes in liver, kidney, testis, ovary and at the same time amelioration of ascorbic acid (vitamin-C) have to observe.

5. MOTIVATION:

At the beginning, hydrogen peroxide was used mainly as the treatment against ectoparasite of adult fish, such as sea lice (*Lepeophtherius salmonis*), or against the microbiota of fish eggs, mainly fungi in freshwater species [28]. Later, it became of interest for microbial control purposes rather than chlorine or antibiotics due to its relatively lower toxicity. Recently, issues concerning system on aquaculture system on the environment and on animal welfare of cultured species have risen.

6. RESEARCH TOOLS AND METHODOLOGY:

Random sampling of fish farm water sample for detection of H_2O_2 [29].

Model animals-

In Present study fish *Catla catla* measuring up to length 12 to 24 cm will be used.

The fish will be divided into four Groups.

- Group-I- Control group
- Group-II- Hydrogen Peroxide (H_2O_2) for 30, 60 and 90 days
- Group-III-Hydrogen Peroxide (H_2O_2) and Vitamin-C for 30, 60 and 90 days
- Group-IV- Vitamin-C for 30, 60 and 90 days.

Experiments of study of the present fish have to be repeated again and again for better results.

Experiments:-

After completion of dosing the animals will be sacrificed on 31st, 61st and 91st days and following parameters will be estimated in different organs.

Parameters:-

1. **a).** Body weight will be taken out at different intervals.

   b). Gonadosomatic Indices will be observed.

2. Histopathological observations in liver, kidney, testis and ovary will be done. These organs will be dissected out and fixed in aqueous Bouin's fluid for histopathological procedures. To observe the histopathological changes in these organs 0.5 mm thick paraffin sections will be cut and stained in Ehrlichs Haemotoxylin and Eosin [30].

3. Biochemical estimations:

- Some enzymological parameters i.e., GOT (Glutamic Oxalic Transaminase), GPT (Glutamic –pyruvic transaminase), ACP (Acid Phosphatase) and ALP (Alkaline Phosphatase) levels in liver and kidney by Reitman and Frankel method [31] and Bergmayers method [32].
- Creatinine test on liver and kidney by Alkaline Picrate method, [33].
- Protein content in Brain, liver, kidney, testis and ovary by Lowry method, [34].
- Cholesterol estimation:- Using Liver, kidney, testis and ovary tissues by Pearson method [35].

For all the biochemical estimations the "P" value will be calculated by student "t" test (Fisher and Yates [36].

Instruments to be used:-

- Microtome
- Refrigerated/Cooling Centrifuge
- Spectrophotometer
- Microscope
- Water bath, Hot Plate etc.

7. SIGNIFICANCE OF PROPOSED WORK:

In view of above investigation there is very special social and economic aspect. The investigation of above noted fish using H_2O_2 as major chemical agents imparts very great benefits to the society as to this the fish is cultivating in ponds, lakes, etc. This fish gives the fish farmers as financial benefits by increasing high yielding capacity which imparts much enjoyable benefits. It indirectly helps the young fish farmers to solve the presents unemployment problem because most of the young generations engage for better growing of the fish or and highly benefits to the downtrodden people of the society. It is also to be noted that our present government should pay special attention for the protection and, large scale production of the fishes.

8. FUTURE PROSPECTS

In this work, we will get to know upto what extent Vitamin-C will ameliorate the Hydrogen Peroxide (H_2O_2) toxicity in different organs (i.e., Brain, Liver, kidney, testis and ovary) of fish *Catla catla.*

9. LIMITATION

Limitation of the present study is the availability of sophisticated well upgraded equipment and apparatus. If time and space provides at a time two or more different species of fishes could be studied for the same.

10. TENTATIVE CHAPTERIZATION:

Chapter 1. Introduction

This chapter will have an introduction in which Hydrogen peroxide (H_2O_2) is a chemical compound, which is used as disinfectant against bacteria and viruses in homes and hospitals. Vitamin C is a water soluble that's found in many foods particularly fruits and vegetables.

Chapter 2. Review of literature

In this chapter, related research work of H_2O_2 and vitamin C is mentioned here.

Chapter 3. Problems to be investigated

This chapter will be on investigation to study the effects of H_2O_2 and Protection of vitamin C in Brain, liver, kidney, testis and ovary on freshwater fish *Catla catla*.

Chapter 4. Objectives and Hypotheses

In this chapter, fish (*Catla catla*) will be selected for study which is easily available for laboratory study. This fish lives in moderate environment and very important dietary fish for the sources for Protein. As for to our knowledge less works or no works available on such type of topic

Chapter 5. Motivation

In this chapter, hydrogen peroxide was used mainly as the treatment against ectoparasite of adult fish, such as sea lice, or against the microbiota of fish eggs, mainly fungi in freshwater species. Later, it became of interest for microbial control purposes or antibiotics due to lower toxicity. Recently, issues concerning system on aquaculture system and on environment.

Chapter 6. Research methodology

In this chapter, Fish *Catla catla* as model animals has chosen. Experimental have to put up in four Groups with different dose and duration. Parameters are histopathological observation, biochemical and enzymological studies has to be estimated. Statistical analysis will be calculated by student “t” test.

Chapter 7. Research Tools

In this chapter, Histopathological studies by Microtome, Biochemical and Enzymological by Refrigerated Centrifuge and Spectrophotometer.

Chapter 8. Significance of proposed work

In this chapter, the investigation of above fish using H_2O_2 as major chemical agents imparts very great benefits to the society as to this the fish is cultivating in ponds, lakes etc., and one of the fast growing fishes in freshwater. This fish gives the much financial benefits to the fish farmers/cultivators by increasing high yielding capacity which indirectly much solves the present unemployment issues to the farmers as well as young generation.

Chapter 9. Concluding remarks

This chapter concludes the results obtained based on our experiment which depends on dose and duration.

REFERENCES:

[1] Housecroft, Catherine E. and Sharpe, Alan G. (2005): Inor ganic Chemistry (2nd ed.). Pearson Prentice-Hall. p. 443. ISBN 0130-39913-2.

[2] Yanong Roy P.E (2018): Use of Hydrogen Peroxide in Finfish Aquaculture. Tropical Aquaculture laboratory school of forest resources and conservation, Program in fisheries and Aquatic sciences, UF/IFAS Extension, Ruskin FL 33570.

[3] Schmidt, Larry J., Gaikowski, Mark, P. and Gingerich, William H. (2006): Environmental Assessment for the use of hydrogen peroxide in Aquaculture for Treating External Fungal and Bacterial. Diseases of cultured Fish and Fish Eggs. USGS science for a changing world, 1-180.

[4] Fadime Eryilmaz Pehlivan (2017): Vitamin C : An Antioxidant Agent, Chapter 2. Intech Open Science pp.23-35.

[5] FAO. (2007). *The state of world Aquaculture and Fisheries 2006*. Food and Agriculture Organization of the United Nations. Fisheries and Aquaculture Department. Rome, Italy.

[6] Worldfish Center. (2002): *Fish: An Issue for Everyone. A Concept Paper for Fish for All, Reprint with revision 2005, 1-10.*

[7] Rach, J.J. , Schrein Theresa M., Howe G.E. and Redman S.D. (1997): Effect of species, life stage, and water temperature on the toxicity of hydrogen peroxide to fish. Progressive Fish-culturist, 59:41-46.

[8] Vera L. M. and Migand H (2016): Hydrogen peroxide treatment in Atlantic salmon induces stress and detoxification responses in a daily manner. Chronobiology international, 33(5):530-42.

[9] Kuznetsov S. V. (1993): The causes of death in animals with hydrogen peroxide poisoning. Biull Eksp Biol Med. 115(6):596-8.

[10] Halliwell B and Gutteridge , J.M.C. (1999): Free radicals in Biology and Medicine, 3rd edn, Clarendon Press, Oxford.

[11] Clement, M-V., Ponton A., and Pervaiz, S. (1998): Apoptosis induced by Hydrogen Peroxide is mediated by decreased superoxide anion concentration and reduction of intracellular milien. FEBS letter, 440(1-2):13-18.

[12] Imlay and Linn, 1987: Mutagenesis and stress responses induced in *Escherichia coli* by hydrogen peroxide, Journal of Bacteriology, 169(7):2967-2976.

[13] Bai J., Rodriguez, A.M., Melendez, J. A. and Cederbaum A. I (1999): Overexpression of catalase in cytosolic or mitochondrial compartment protects HepG2 cells against oxidative injury. Journal of Biological Chemistry, 274(37):26217-26224.

[14] Halliwell B and Gutteridge , J.M.C. (1999): Free radicals in Biology and Medicine, 3rd edn, Clarendon Press, Oxford.

[15] Matsumoto A., Okado, A., Fuju T., Fujii J., Egashira M., Nikawa N. and Taniguchi N. (1999): Cloning of peroxiredoxin gene family in rats and characterization of the fourth member. FEBS letter, 443(3):246-250.

[16] Chances B., Sies H., and Boveris A. (1979): Hydroperoxide metabolism in mammalian organs. Physiological Reviews. 59(3):527-605.

[17] Mirvaghefi, A. Ali, M. and Poorbagher, H. (2016): Effects of vitamin C on Oxidative stress parameters in rainbow trout exposed to diazinon. Ege Journal of Fisheries and Aquatic sciences, 33(2):113-120.

[18] Buettner, G.R. & Moseley, P.L. (1993). EPR spin trapping of free radicals produced by bleomycin and ascorbate. Free radical research communications, 19: S89-S93. doi:10.3109/10715769309056s89 .

[19] Deshpande, S.S., Deshpande, U.S. & Salunkhe, D.K. (1996): Nutritional and health aspects of food antioxidants. In: Madhavi DL, Deshpande SS, Salunkhe DK (EDs.) Food antioxidants, technological, toxicological, and health perspectives, pp 361-470. New York: Marcel Dekker.

[20] Steinberg D (1997): Low density lipoprotein oxidation and its pathobio- logical significance. J Biol Chem 272:20963–20966.

[21] Steinberg D, Lewis A (1997): Conner Memorial Lecture. Oxidative modification of LDL and atherogenesis. Circulation 95:1062– 1071.

[22] Chisolm GM, Steinberg D (2000): The oxidative modification hypothe- sis of atherogenesis: an overview. Free Radic Biol Med 28:1815– 1826.

[23] Crawford RS, Kirk EA, Rosenfeld ME, LeBoeuf RC, Chait A (1998): Dietary antioxidants inhibit development of fatty streak lesions in the LDL receptor-deficient mouse. Arterioscler Thromb Vasc Biol 18:1506–1513.

[24] Mahfouz MM, Kawano H, Kummerow FA (1997): Effect of cholesterol- rich diets with and without added vitamins E and C on the severity of atherosclerosis in rabbits. Am J Clin Nutr 66:1240– 1249.

[25] Mohamed, Shaymaa H., Ebtihal M. M. and Leithy, L.E. (2019): Molecular, biochemical and histopathological studies on the ameliorative effect of vitamin C on the renal and muscle tissues of Nile tilapia fish (*Oreochromis niloticus*) affected by the usage of engine oil. Aquaculture Research. 50:3357–3368.

[26] Uğur Yavuz, and Yusuf Bozkurt (2020): Protective Effect of Vitamin C (Ascorbic Acid) Supplementation on Post-Thaw Motility and Fertility of Cryopreserved Rainbow Trout (*Oncorhynchus mykiss*) Sperm. Turkish Journal of Agriculture – Food Science and Technology, 8(6): 1348 – 1352.

[27] Wang, L., Chen, Q., Zhuang, S., Wen, Y., Cheng, W., Zeng, Z., (2020): Effec tof Anoectochilus roxburghii flavonoids extract on H2O2 - Induced oxidativestress in LO2 cells and D-gal induced aging mice model. J. Ethnopharmacol. 254, 112670.

[28] Papiol G.G. and Rogue A. (2013): Uses of Hydrogen peroxide in aquaculture, Hydrogen Peroxide: Detection, Application on Health Implications, 153-170.

[29] APHA Standard method for examination of water and waste water, 1998. 20th edition, American Public Health Association, Washington DC.

[30] Ehrlich,P. (1986): Fragkaster Zeitshift Fur Wissenschfliche Microskopic and Fur Mikroscopicha Technik, 3:150.

[31] Reitman, S., and Frankel, S.A. (1957): A calorimetric method for the determination of serum glutamate oxaloacetate and glutamate pyruvate transaminase. *An.J. Clin. Pathol. K.,* 28:56-63.

[32] Bergmeyer, H.U., Bernt, E. and Hess, B. (1963): Lactate dehydrogenase, in methods of enzymatic analysis. H.U. Bergmeuyer, (Ed). London: *Academic Press* 2:735.

[33] Alkaline Picrate method, (1945): For determination of Creatinine in serum and Urine. *J.Biol. Chem.,* 158:581.

[34] Lowry, O.H., Rosenbrough, M.J., Farr, A.L and Randall, R.J. (1951): Protein measurement with Folin-phenol reagent. *J. Biol. Chem.,* 193:265-275.

[35] Pearson, S., Stern, S. and McGauak, T.K. (1953): A rapid accurate method for the determination of total Cholesterol in serum. *Analytical chemistry*, 25:813-814.

[36] Fisher, R.A. and Yates, F. (1953): Statistical Tables. London: *Oliver and Boyed*.